Current status of A_1 adenosine receptor allosteric enhancers

Romeo Romagnoli*[a], Pier Giovanni Baraldi [a], Allan R. Moorman[b], Pier Andrea Borea[c], Katia Varani[c]

[a]Dipartimento di Scienze Chimiche e Farmaceutiche, Università di Ferrara, Italy; [b]Alta Vetta Pharmaceutical Consulting, L.L.C., Durham, North Carolina; [c]Dipartimento di Scienze Mediche, Sezione di Farmacologia, Università di Ferrara, Italy

Correspondence should be addressed to

Prof. Romeo Romagnoli, Dipartimento di Scienze Chimiche e Farmaceutiche, Via Fossato di Mortara 17-19, 44121, Università di Ferrara, Ferrara, Italy. Phone: 39-(0)532455303. Fax: 39-(0)532455953. E-mail: rmr@unife.it

Abstract

Adenosine is an ubiquitous nucleoside involved in various physiological and pathological functions by stimulating A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors (ARs). The pharmacological role of A_1ARs is well known in physiological conditions and in a variety of pathologies, primarily including central nervous system diseases or cardiac disorders. Allosteric enhancers to A_1ARs may represent novel therapeutic agents because they increase the activity of these receptors by mediating a shift to their active form in the A_1AR-G protein ternary complex. In this manner, they are able to amplify the action of endogenous adenosine, which is produced in high concentrations under conditions of metabolic stress, such as ischemia or hypoxia. A_1AR allosteric enhancers could be used as a justifiable alternative to the exogenous agonists that are characterized by receptor desensitization and down regulation. In this review an analysis of the structure-activity relationships of some of the most interesting allosteric modulators of A_1ARs and their potential pharmacological roles has been reported.

Introduction

Adenosine is an ubiquitous endogenous nucleoside modulator playing a fundamental role in a wide variety of physiological processes and produced rapidly during hypoxic, ischemic, and inflammatory events [1]. This nucleoside is considered to exert cardio- and neuro-protective effects mediated by interactions with four different G-protein coupled adenosine receptors (ARs), named A_1, A_{2A}, A_{2B}, and A_3, which are widely distributed throughout the body [2]. These receptors differ in their affinity for adenosine, in the type of G proteins recruited, and in their respective cellular signaling pathways. In particular, A_1 and A_3ARs are coupled to members of an inhibitory family of G-proteins (Gi), inducing the inhibition of adenylate cyclase and the reduction of intracellular cyclic adenosine monophosphate (cAMP). The A_{2A} and A_{2B} receptors preferentially are coupled to Gs proteins that stimulate adenylate cyclase activity [3]. A_1ARs also activate phospholipase-Cβ, which increases inositol 1,4,5-triphosphate (IP_3) and intracellular calcium [4]. These receptors are coupled to pertussis toxin-sensitive potassium channels, inhibit voltage-gated calcium channels, and modulate extracellular signal regulated protein kinases (ERKs) [5].

A variety of effects mediated by adenosine occur via A_1ARs, extensively expressed in the central nervous system (CNS), with high levels in brain, cortex, cerebellum, hippocampus, and dorsal horn of the spinal cord, and in other tissues such as kidney, lung, bladder, and heart [6]. In particular, A_1ARs modulate the activity of the CNS at the cellular level and are responsible for sedative, anticonvulsant, anxiolytic and locomotor depressant effects induced by adenosine [7]. It also has been reported that A_1AR modulation by adenosine mediates analgesia by the inhibition of nociceptive neurons [8]. Stimulation of A_1ARs in the heart exerts a cardioprotective effect by inhibiting norepinephrine release from sympathetic nerve endings. Moreover, adenosine can protect coronary tissues through its effect in ischemic preconditioning, a brief period of ischemia and reperfusion, which can protect myocardium against infarction during a subsequent prolonged ischemic insult [9]. Different effects are mediated by the selective activation of A_1ARs, including

hypotension, neuroprotective effects produced during hypoxic and ischemic conditions, reduction of neuropathic pain, reduction of lipolysis in adipose tissue, vasoconstriction, inhibition of renin secretion, diuresis, and natriuresis in the kidney [10]. Due to the ubiquitous presence of ARs, the selective modulation of A_1ARs within specific tissues has attracted much attention as a potential therapeutic approach [11]. It is well known that A_1AR agonists could be used potentially as analgesics, antiepileptics, neuroprotective, anti-arrhythmic, and anti-lipolytic agents [12]. Unfortunately, the development of full A_1AR agonists as therapeutic agents has been limited by side effects in tissues other than the therapeutic target, such as severe hypotensive action, heart block, impairment of renal function, and the propensity of agonists to cause receptor desensitization upon prolonged exposure [13]. Allosteric modulation may provide an alternative approach to highly selective agonists for A_1AR [14]. The advantage of allosteric enhancers is related to their ability to induce responses only under conditions in which the endogenous agonist is able to exert its physiological effects. The problem of side effects associated with the use of A_1AR agonists might be resolved by the use of allosteric enhancers acting synergistically with endogenous adenosine, which is produced in high concentrations under hypoxic or anoxic conditions [14].

An allosteric enhancer (AE) is a molecule that interacts with a specific locus on a receptor named the allosteric binding site, topographically and physically distinct from the orthosteric site [15-16]. Allosteric modulators can induce either a negative or positive effect on cellular signalling and receptor coupling, defining selective negative or positive allosteric modulators, respectively [17]. Compounds that are able to interact with both the orthosteric and allosteric sites, referred to as bitopic ligands, also have been reported [18]. From a pharmacological point of view, the advantage of AEs is related to their ability to selectively tune responses only under conditions in which the endogenous agonist concentration is sufficient to exert its physiological effects [19]. As a consequence, AEs show a number of advantages as potential drugs, primarily due to their selectivity for individual receptor subtypes. Because the allosteric binding sites are not subject to evolutionary

pressure to remain conserved across receptor families, they are likely to display greater divergence than the orthosteric sites [18, 19]. The binding of an AE with the A_1AR may modify either the affinity or efficacy or both parameters mediates a significant increase in the affinity and potency of the endogenous agonist or an exogenous synthetic agonist [20, 21]. Several studies have attempted to provide credible evidence regarding the exact location of the allosteric site and the amino acids involved in the receptor binding of the allosteric ligands. Molecular modeling studies have suggested that the A_1AR allosteric site is located in proximity to the orthosteric site, possibly within the boundaries of the second extracellular loop of the receptor [19]. A study using bitopic ligands suggested that the extracellular loop 2 (ECL2) of A_1AR represents an AE binding region because mutations in these residues are able to modify the AE activity [22]. It is possible to hypothesize that AEs function by occupying the identified ECL2 vestibule, impeding agonist dissociation and providing a very interesting opportunity to use structural data to guide the development of new AEs for A_1AR [23]. Site-directed mutagenesis studies have also been performed in order to identify the amino acids involved in the binding site of A_1AR allosteric modulators [24].

The identification of the molecular determinants of AE activity and mathematical modeling could be used to gain mechanistic insights into allosteric enhancer AEs [25]. The models involve simplified systems encompassing the receptor, the orthosteric ligand, and the allosteric enhancer AEs. Such modeling predicted that the A_1AR allosteric site resides along the path followed by a ligand to reach the orthosteric site [26]. It should be noted that AE activity has been reported to vary, both *in vitro* and *in vivo*, across species such as human, mouse, guinea pig. This notion is supported by the observation that AE binding sites have not been precisely determined because the allosteric sites are not closely conserved between G-protein coupled receptor subfamilies [27]. However, many investigations of the AE activity did not distinguish enhancer activity from competitive antagonist activity. Consequently, the measured activities were a composite of allosteric and competitive antagonist effects [28]. However, many investigations of the AE activity

did not distinguish enhancer activity arising from interaction at an allosteric site from activity arising from a conformational change affecting the orthosteric site or dualsteric binding at the orthosteric and allosteric sites.. Consequently, the measured activities were a composite of allosteric and orthosteric effects [28].

In the present review the most important A_1AR allosteric modulators and novel enhancer molecules published in the last five years have been described from a chemical and pharmacological point of view. It is now apparent that A_1AR allosteric enhancer AEs may represent novel therapeutic agents in the treatment of diseases based on hypoxia and ischaemia-induced injury, cardiac arrhythmia, lipolysis, and in neuropathic pain, providing an interesting approach to improved drug responses.

Structure activity relationship (SAR) and chemical structures of A_1AR allosteric modulators

From a pharmacological point of view, AE activity can be characterized by using different experimental approaches, as indicated by the score determination by using: a) equilibrium binding of the agonist, [^{125}I]ABA ([^{125}I]-N^6-(4-aminobenzoyl)adenosine) to the A_1AR; b) addition of the candidate allosteric enhancer AE to stabilize the agonist-receptor-G protein ternary complex; c) dissociation of the complex by adding a combination of a large excess of A_1AR antagonist and GTPγS, to accelerate agonist radioligand dissociation. A score of 100% indicates that the AE completely blocks radioligand dissociation and a score of zero corresponds to a compound that has no effect on dissociation of the radioligand. Other methods can be used in the characterization of AEs, such as performing saturation binding experiments in the absence or presence of the AE to indicate an alteration in the A_1AR affinity or in the receptor density. Competition binding experiments of an A_1AR agonist in the absence or presence of AEs could be very useful as well, to verify the effect of the AE on the affinity value of the receptor for the agonist. The effect of AEs on cAMP production has been used to demonstrate the capability of these compounds to affect Gi proteins, thereby inhibiting adenylate cyclase activity.

2-Amino-3-substituted-4,5-dialkylthiophene analogues

PD 81,723 [(2-amino-4,5-dimethylthiophen-3-yl)(3-(trifluoromethyl)phenyl)methanone, **1**] (Figure 1) was the first allosteric modulator of the A_1AR, discovered by Bruns et al. in 1980 while screening the Parke-Davis compound library. The compound was a chemical intermediate in the synthesis of new benzodiazepine-like compounds [29,30]. This compound was shown to selectively increase the binding and slow the dissociation of the agonist radioligand [^{3}H]N^6-cyclohexyladenosine ([^{3}H]CHA) from the A_1AR in rat brain membranes. Among the compounds evaluated, it was reported to have the best ratio of enhancer to antagonist activity [31]. Among the compounds evaluated, it was reported to have the best ratio of AE activity to an antagonistic activity that could be attributed to a conformational change of the receptor site or dualsteric binding of the AE to the allosteric and orthosteric sites [31]. PD 81,723 was found to stabilize the high-affinity conformational state of the A_1AR-G-protein complex and became the prototypical allosteric modulator of the A_1AR [32,33]. This compound has been extensively modified structurally at the 3-, 4-, and 5-positions, leading to a diverse number of substituted derivatives. Several review articles have reported the wide range of 2-amino-3-aroylthiophene derivatives modified at either the C-4 or C-5 positions of the thiophene ring as highly potent and selective AEs at the A_1AR [34-45].

Figure 1 and Table 1 should be inserted here

In the series of 2-amino-3-benzoylthiophene derivatives, SAR studies indicated that the benzoyl group was essential for activity and lipophilic substituents at either the *meta* or *para*-position on the phenyl of the benzoyl moiety (such as 3-CF_3, 4-CH_3, 3,4-Cl_2 or 4-*tert*-butyl) favoured high AE activity. Activity can be greatly increased by appropriate thiophene 4-alkyl substitution, or by the presence of a polymethylene bridge linking the C-4 and C-5 positions, with potency increasing in proportion to the length of the polymethylene chain. Bulky substitution at the C-5 position,

combined with hydrogen or small alkyl substitution (methyl or ethyl) at the C-4 position was shown to favour antagonist activity. The 2-amino group is essential for AE activity, and an intramolecular hydrogen bound with the carbonyl oxygen of the benzoyl moiety has been hypothesized to contribute to activity. Several studies have emphasized the presence of two regions in the allosteric binding site of the hA_1AR: one region is able to accommodate the 2-amino-3-aroyl thiophene scaffold, while the second wide lipophilic domain interacts with substituents at C-4 and C-5 of the thiophene ring [41, 43-45].

Starting from PD 81,723, van der Klein et al. have identified novel 2-amino-3-benzoylthiophene derivatives, finding compound **2** (T-62) and **3** (LUF 5484) to be more potent than the reference compound **1,** but with comparable antagonist activity ability to displace antagonists, due to a dualsteric behaviour (binding to both orthosteric and allosteric sites) or a conformational shift of the receptor site due to binding at the allosteric site [46]. The biological assays were performed on rat brain cortex membranes. The enhancer activity, expressed as a decreased dissociation of radiolabeled agonist in the presence of 10 μM AE, was 123% and 151% for compounds **2** and **3**, respectively, and superior to that of PD 81,723 (Table 1). Compound **2** was found to be efficacious when tested in animal models of neuropathic pain associated with hyperalgesia and allodynia [47-49]. The compound was progressed into Phase II clinical trials for evaluation of the safety and analgesic efficacy in subjects with postherpetic neuralgia and its associated pain (King Pharmaceuticals). Those trials were halted after some subjects experienced transient elevations in liver enzymes [50].

A further series of 2-amino-3-benzoyl thiophene derivatives was developed by Baraldi et al., with compound **2** and its *p*-bromobenzoyl analogue **4** found to be more potent than PD 81,723, each causing a significant reduction of cAMP production at low concentration (0.1 μM) in Chinese hamster ovary (CHO) cells stably transfected to express the recombinant human A_1ARs (hA_1CHO) [51]. By systematic modification of PD 81,723, Tranberg et al. identified a new series of 2-amino-

3-aroyl-4,5-dialkyl thiophene derivatives, with the *m*-methoxybenzoyl analogue **5** as the most potent compound of the series, showing an high allosteric enhancer AE score (99%) and relatively low antagonist potency ability to effect a decrease in [^{3}H]CPX binding (13%) at 100 μM. In the same experimental assay, the allosteric enhancer AE score for **1** was 19% at the same concentration [52]. Nikolakopoulos et al. studied 2-aminothiophene 3-carboxylates and carboxamides to explore the effect of substituents at C-3 of the thiophene on AE activity. The 2-aminothiophene-3-carboxylate derivative **6** was the most potent compound of the series, with an allosteric enhancer AE score of 79.5% (28% for PD 81,723) and a percentage inhibition of [^{3}H]CPX binding that was one-half that of PD 81,723 activity as a competitive antagonist that was one-half that of PD 81,723 in a [^{3}H]CPX binding assay [53]. Compound **6** represented the first example of an AE with a polar substituent at the C-3 position of the thiophene ring, in contrast with the paradigm that a lipophilic substituent at C-3 was essential for activity. Baraldi et al. investigated the replacement of the 3-benzoyl with a more lipophilic 3-naphthoyl moiety [54]. In this series of 2-amino-3-naphthoyl thiophene derivatives, compound **7** proved to be the most efficacious compound, increasing agonist binding to hA$_1$CHO, brain, and rat cortex membranes by 149%, 43% and 27%. These data can be compared with those obtained with the reference compound **1** (51%, 15% and 22%, respectively). Replacement of the phenyl of the benzoyl moiety at the C-3 position with a 3-thienyl maintained allosteric enhancer AE activity, with the 2-amino-3-heteroaroyl thiophene derivative **8** showing activity comparable to PD 81,723 in a cAMP assay with hA$_1$CHO cells [55]. Recently, Butcher et al have reported that compound **9** (VCP333), an analogue of T-62 with a 7-membered heterocyclic ring fused to the thiophene, may improve functional recovery in mouse hearts following an ischaemic injury [56].

2-Amino-3-aroyl-4-arylthiophene analogues

Lutjens et al. reported the first examples of 2-aminothiophene derivatives with additional 3-aroyl substituents and characterized by large hydrophobic groups at either the 4- or 5-positions of the thiophene ring (Figure 2). For the 3-benzyloxycarbonyl and 3-naphthoyl derivatives **9** **10** and **10**, **11** the allosteric enhancer AE score was 63% and 52%, respectively (19% for PD 81,723) [5657]. In the series of 3-benzoyl derivatives, the 4,5-diaryl thiophene substitution reduced the AE activity, which was increased removing the 5-aryl group. For comparison, derivative **11**12 showed an allosteric enhancer AE score of 18%, while the analogue **12**13**,** with no 5-substituent was shown to be more efficacious, with an AE score of 77% [5758]. Aurelio et al. identified an optimized combination of aroyl and aryl substitutions at the 3- and 4-positions of the 2-aminothiophene scaffold with the 4-[3,5-bis(trifluoromethylphenyl)]-2-amino-3-benzoyl thiophene derivative **13**14**.** Reported as the most efficacious compound of the series in a functional assay of A_1AR-mediated phosphorylation of ERK1/2 in CHO cells, they identified both potential agonist effects as well as robust allosteric enhancer AE activity, modulating the activity of the othosteric agonist, R-PIA [5859].

Figure 2 should be inserted here

From compound **12**13, the corresponding 5-methyl analogues **14**15 and **15**16**,** differing only in terms of the presence and absence of the electron-withdrawing chloro group at the *para*-position on the benzoyl moiety, were identified as biased allosteric agonists and positive allosteric modulators of the A_1AR agonist function in two different functional assays of ERK 1/2 phosphorylation and inhibition of cAMP production in CHO cells [5960]. Aurelio et al. demonstrated that 2-aminothiophene derivatives with an ethoxycarbonyl group in the 3-position, combined with 4,5-diaryl substitution supported AE activity, which was substantially decreased by replacing the ester with an aroyl moiety. In the 3-ethoxycarbonyl series, the 4-(*m*-trifluoromethylphenyl)-5-(*p*-chlorophenyl) analogue **16**17 was the most potent and efficacious derivative, with an AE score of

57%. Unfortunately, **16**17 also showed a greater antagonist activity tendency to inhibit antagonist binding than PD 81,723 in a [^{3}H]CPX competitive binding assay, with 58% and 18% inhibition, respectively [5758]. Replacement of the ethoxycarbonyl moiety at the 3-position of the 2-aminothiophene scaffold by an amide or hydrazide functionality led to a substantial reduction of AE activity [6061]. It has been confirmed that in the series of 3-aroylthiophene derivatives, the analogues with a 4-aryl substituent were found to be AEs, while the introduction of an additional aryl group at the 5-position (such as in compound **11**12) reduced the potency. A contrasting situation was observed for the 3-ethoxycarbonyl derivatives, with the 4,5-diaryl analogues, which were more active than the corresponding 4-aryl counterparts.

2-Amino-3-(4-chlorobenzoyl)-4-[(4-(aryl)piperazin-1-yl)methyl]thiophene derivatives.

The importance of having an appropriate hydrophobic substitution at the 4-position of the thiophene ring for potent AE activity at the A_1AR was confirmed by the preparation of a series of 2-amino-3-(4-chlorobenzoyl)-4-[(4-(aryl)piperazin-1-yl)methyl]thiophene derivatives with general structure **17**18 (Figure 3). The degree of allosteric enhancement was influenced by the number and position of electron-withdrawing or electron-releasing groups (EWGs or ERGs, respectively) on the phenyl tethered to the piperazine moiety. Among them, the 4-chloro (**17a**18a), 4-trifluoromethyl (**17b**18b), 3,4-difluoro (**17c**18c)**,** 3-chloro-4-fluoro (**17d**18d) and 4-trifluoromethoxy (**17e**18e) derivatives were reported to be the most active compounds in saturation and competition binding studies [61, 6262, 63]. In [^{3}H]CCPA saturation experiments, the Bmax shift, expressed as an X-fold increase, was 1.3 for PD 81,723, but ranged from 6.8 to 7.7 for compounds **17a-e**18a-e. In [^{3}H]DPCPX competition binding experiments, the CCPA Ki shift, also expressed as an X-fold increase, was 1.7 for PD 81,723 and between 5.3- to 6.3-fold for compounds **17a-e**18a-e (Table 1 2). Thus, the enhancers were able to mediate a shift of the A_1ARs towards the high affinity state, as suggested by the increase of CCPA affinity in the presence of an AE with respect to the control condition.

Interestingly, the presence of the AEs mediated an increase in Bmax shift derived from [^{3}H]CCPA saturation binding experiments, suggesting the ability of these novel compounds to mediate a shift from the ground state (R) to the activated state (R*) of the A_1ARs [6263]. None of the examined compounds at a concentration of 10 μM significantly inhibited the specific binding of the radioligands to [^{3}H]DPCPX, [^{3}H]ZM241385 and [^{3}H]MRE3008F20 to human A_1, A_{2A} and A_3ARs, respectively. These very interesting data suggest that the A_1AR enhancers were not able to bind to the orthosteric site, even though they displayed good enhancer activity.

Figure 3 and Table 12 should be inserted here

The results obtained with compounds **17a-e18a-e** make it possible to identify in the phenylpiperazine moiety a portion of the molecule that is important for interaction with the allosteric binding site of A_1ARs and whose manipulation contributes to improved AE activity. It may be possible that the phenyl ring of the phenylpiperazine moiety contributes to the activity via a π–π interaction with specific amino acid residues in the allosteric binding pocket of the A_1AR. Based on these findings, in the search for more potent and efficacious compounds with reduced competitive antagonist activity, we investigated the structural modification at the 5-position of 2-amino-3-(4-chlorobenzoyl)-4-[(4-(aryl)piperazin-1-yl)methyl]thiophene derivatives **17a-e18a-e**, replacing the hydrogen at the 5-position with bromine, small alkyl groups (methyl and ethyl) and a phenyl ring with electron-withdrawing fluorine and chlorine groups. Comparing compounds characterized by the presence of the same arylpiperazine moiety linked through a methylene at the thiophene 4-position, the 5-aryl substituted derivatives were more potent than the alkyl and bromo counterparts. The 5-aryl thiophene analogues **18-2019-21** were the most active compounds of the series in binding (saturation and displacement) assays (Figure 4). In [^{3}H]CCPA saturation binding experiments, compounds **1819**, **1920** and **2021** induced a Bmax shift (X-fold increase) of 14.1, 11.2 and 11.8, respectively, while in [^{3}H]DPCPX competition binding experiments, compounds **1819**,

1920 and **2021** increased the apparent affinity (Ki) of CCPA approximately 13.3-, 10.5- and 13.0-fold, respectively, resulting in compounds that were almost 5- to 6-fold more active than PD 81,723 [6364]. Saturation and competition binding experiments also demonstrated that the 5-aryl substituted derivatives **18-2019-21** were more active than the corresponding 5-unsubstituted analogues **17a-e18a-e.** Compound **1819**, in different mouse models of acute and chronic pain, effectively inhibited nociceptive responses in the formalin and writhing tests, with effects comparable to those of the reference analgesic morphine. Compound **1819** was anti-allodynic in the neuropathic pain model and did not display locomotor or cataleptic side effects. This study clearly demonstrated the antinociceptive effects of the novel compound **1819**, one of the most potent and effective A_1AR positive allosteric modulators synthesized thus far [6465].

Figure 4 should be inserted here

Encouraged by the results obtained with the arylpiperazine derivatives **17a-e18a-e**, Romagnoli et al continued to follow the strategy of optimization of the substituent attached to the methylene at the thiophene 4-position, replacing the arylpiperazine moiety with a series of fused indole nuclei, to furnish the corresponding 1,2,3,4-tetrahydropyrazino[1,2-*a*]indole and 1,2,3,4,10,10a-hexahydropyrazino[1,2-*a*]indole derivatives **21a-c22a-c** and **2223**, respectively, as the most active compounds of the whole series in saturation and competition assays [6566]. The pyrazino[1,2-*a*]indole system might be viewed as a conformationally restricted arylpiperazine, in which the *ortho*-position of the phenyl ring was tethered to the carbon at the 2'-position of the piperazine moiety by a methylene unit. Compounds **21a22a**, **21b22b**, **21c22c**, and **2223** caused a Bmax shift of more than 7-fold (7.5, 8.2, 7.4, and 8.1, respectively), while in competition binding experiments derivatives **21a22a**, **21b22b**, **21c22c**, and **2223** enhanced the apparent affinity (Ki) of CCPA approximately of 6.6-, 7.2-, 6.9-, and 7.6-fold, respectively. Biological data confirmed that

replacement of the arylpiperazine moiety by a tetrahydro- or hexahydro-pyrazino[1,2-*a*]indole nucleus in the 4-position of thiophene ring is well tolerated.

2-Amino-3-(4-chlorobenzoyl)-4-neopentyl-5-substituted thiophene derivatives

To further study the role of various alkyl substitutions at the 4-position of the 2-amino-3-(4-chlorobenzoyl)thiophene nucleus, Romagnoli aet al. also synthesized a novel series of 2-amino-3-(4-chlorobenzoyl)-4-neopentylthiophene derivatives with variable modifications (bromine, aryl, heteroaryl, aryl/heteroarylacetylene) at the 5-position of the thiophene ring (Figure 5) [66, 67 67,68]. Among the C-5 aryl derivatives, the *meta*-methoxyphenyl and *para*-tolyl derivatives **23a24a** and **23b24b** were the most active compounds in binding assays. Compounds **23a24a** and **23b24b** caused a Bmax shift to hA_1AR of 6.3- and 6.4-fold, respectively, when tested at 10 μM concentration in [^{3}H]CCPA saturation binding experiments (Table 2). The same derivatives **23a24a** and **23b24b** enhanced the apparent affinity of CCPA by 5.8- and 6.0-fold, respectively, being twice as active as the corresponding 5-unsubstituted derivatives. In the series of 4-neopentyl derivatives, the presence of an acetylene spacer between the 5-position of the thiophene ring and a (hetero)aryl moiety improves the allosteric enhancer AE activity, while reduction of the acetylene to a flexible ethylene spacer decreased the activity [6768]. The existence of a neopentyl group at the 4-position and a variably substituted (hetero)arylacetylene moiety at the 5-position of 2-amino-3-(4-chlorobenzoyl)thiophene skeleton represented the best combination, to afford a series of compounds with improved AE activity. while favouring the expression of allosteric properties over orthosteric antagonist properties. The phenylethynyl (**23c24c**), *p*-tolylethynyl (**23d24d**), 2,4,5-trimethylphenylethynyl (**23e24e**), and *p*-methoxyphenylethynyl (**23f24f**) derivatives were the most promising compounds in binding (saturation and competition) studies, being able to potentiate agonist [^{3}H]CCPA binding to theA_1AR, with **23c24c** as the best compound of the series. From the hA_1AR receptor density (Bmax) calculated in the presence and absence of these compounds, the

derivatives **23c-f24c-f** caused a Bmax shift of more than 7-fold, with **23c24c** causing more than a 9-fold shift. In hA_1CHO cell membranes, using [^{3}H]DPCPX as radioligand, the compounds **23c24c**, **23d24d**, **23e24e**, and **23f24f** augmented the apparent affinity of CCPA approximately 10.0-, 7.5-, 7.5-, and 8.2-fold, respectively, being at least 1.5-fold more active than the 5-aryl derivative **23b24b**. In association and dissociation experiments, derivative **23c24c** significantly retarded the dissociation rate of the agonist [^{3}H]NECA from the A_1AR by approximately 2-fold, providing an apparent increase in affinity. The association rate of [^{3}H]NECA was not significantly influenced by the presence of **23c24c**, however. The functional effects of **23a-23f24a-f** have been investigated by using cAMP assays performed in hA_1CHO cells. where a significant reduction of the production of cAMP was observed (Figure 6). Interestingly, these compounds at 100 nM concentration were able to inhibit cAMP production more than PD 81,723 at the same concentration, suggesting their excellent capacity to reduce cAMP levels.

Figure 5 should be inserted here

Future perspectives

Allosteric modulators for the A_1AR have been the most studied among the ARs and several drug candidates have been identified as a promising and innovative approach alternative, alone or in combination with other therapeutic agents, for the treatment of neuropathic pain. From the discovery of PD 81,723, extensive modifications of this compound have generated multiple series of analogues with improved potency and efficacy and reduced competitive antagonist activity. Although it appeared possible to separate allosteric enhancer activity at the A_1-AR from properties that would reduce antagonist binding, these molecules should also be evaluated for their selectivity for the allosteric site of the A_1-AR, to demonstrate that they do not also act as allosteric enhancers of the A_{2A} and A_3 adenosine receptors.

All of the compounds under evaluation possess a 2-amino group and a benzoyl substituent in the 3-position, The point of divergence for the different series is in the substituents at the 4/5 positions of the thiophene core, with various alkyl and aryl substituents that are tolerated at the 4- and 5-positions of the thiophene core. The amino and carbonyl group of the benzoyl moiety are fundamental for AE activity. Any modification of the amino function has resulted in a reduction of activity. Substituent size at the thiophene C-4 position appears to be a factor closely related to activity, with the 4-neopentyl substitution showing the greatest enhancement in activity among the alkyl substituents. Encouraging results have been obtained with compound **18**, characterized by the presence of a phenylpiperazine moiety at the 4-position of the 2-amino-3-(*p*-chlorobenzoyl)-5-(*p*-fluorophenyl)thiophene nucleus. Additional studies of metabolic stability and lack of toxicity will be required to determine if this compound could be useful as a therapeutic agent.

The therapeutic potential of many of the currently available selective AEs for the A_1AR appears to be hampered by the fact that these molecules also possess antagonist properties against this receptor subtype at higher concentrations. The therapeutic potential of many of the currently available selective AEs for the A_1AR appears to be hampered by the fact that these molecules also possess an ability to inhibit antagonist binding at this receptor subtype at higher concentrations, possibly due to a conformational shift of the receptor following AE binding or a dualsteric activity. The most recently published molecules clearly showed a separation of these orthosteric and allosteric activities. However, the 2-aminothiophene system may not be the ideal system for drug development, being a potential carcinogen and not stable in solution. Replacement of the 2-aminothiophene molecular system with new scaffolds, such as 2-aminothiazole [6869], 2-aminothieno[3,4-*d*]pyridazine [6970] and 1,2,4-thiadiazole [7071], failed to furnish allosteric modulators of A_1AR with improved potency and reduced competitive antagonist activity compared to existing aminothiophenes a reduced ability to affect antagonist binding, compared to existing aminothiophenes.

Executive summary

- Adenosine is an endogenous nucleoside modulator released from almost all cells and is generated in the extracellular space by ATP breakdown.
- A_1ARs are widely distributed throughout the body, showing a high density in CNS and cardiac tissues.
- A_1AR belongs to the G_i protein-coupled receptors, and primarily mediates the inhibition of adenylyl cyclase with a decrease of intracellular levels of cAMP.
- A_1AR is responsible for several physiological effects of adenosine in various tissues and cell types such as the brain, heart, kidney and adipocytes.
- Highly selective full agonists to A_1AR have unwanted side effects that limit their clinical application.
- Allosteric modulators may have a therapeutic potential due to their ability to increase the efficacy of the endogenous agonist, adenosine.
- Allosteric enhancers may permit the use of exogenous agonists to be avoided, thereby reducing the side effect profile..
- A_1AR enhancers offer the prospect of increasing receptor selectivity where it has not been possible to design selective orthosteric drugs without side effects.

KEY TERMS

Allosteric enhancer

A ligand that increases the affinity and efficacy of the orthosteric agonist by combining with a distinct site, named allosteric, on the receptor, while having no effect on his own.

Allosteric site

A ligand-binding allosteric site that is distinct from the orthosteric binding site.

Orthosteric site

The binding site for the endogenous or synthetic agonists.

Allodynia

Pain generated by a stimulus that is not normally painful.

Table 1. Allosteric enhancer data compounds **1-6** and **10-17**.

Compound	Enhancement[a]		AE score[b]	Activity[c]	
	%	EC_{50} (μM)	%	3 μM	10 μM
1 (PD 81,723)	100	15			
2	123	6.8			
3	151	6.2			
4	128.2	16.4			
5			99[d]		
6			79[e]		
10			63[d]		
11			52[d]		
12			18[e]		
13			77[e]		
14				83±7	93±8
15				77±6	73±3
16				94±3	94±4
17			57[e]		

[a]Enhancing activity (at 10 μM of tested compound) is expressed as % decrease (±SEM) in [^{3}H]CCPA dissociation over control (0%) and that of PD 81,723 (100%) (ref. 46).
[b]The assay on CHO-K1 membranes stably expressing hA_1ARs using allosteric enhancers at 100 μM (ref. 59, 60).
[c]Effect of the compounds **14-16** at 3 and 10 μM on A_1AR-mediated stimulation of ERK 1/2 phosphorylation in CHO cells in the presence of R-PIA.
[d] The AE score for PD 81,723 was 19% (ref. 52, 57).
[e] The AE score for PD 81,723 was 28% (ref. 53, 58).

Table 1 2. A_1AR density, expressed as Bmax values, obtained by [^{3}H]CCPA saturation binding assays in hA_1CHO membranes in the presence of PD 81,723 and of allosteric enhancers (10 μM) and the relative calculated Bmax shift (A). Modulation of CCPA affinity (CCPA Ki shift) by the same compounds (10 μM) in [^{3}H]DPCPX competition binding experiments (B).

Compound	(A) [^{3}H]CCPA saturation binding experiments		(B) [^{3}H]DPCPX competition binding experiments	
	Bmax (fmol/ mg protein)	Bmax shift (fold of increase)	CCPA Ki (nM)	CCPA Ki shift (fold of increase)
PD 81,723	680±59	1.3±0.1	9.8±0.9	1.6±0.1
17a18a	3626±332	7.0±0.6	2.7±0.3	5.6±0.5
17b18b	3989±377	7.7±0.7	2.4±0.2	6.3±0.5
17c18c	3708±384	7.2±0.6	2.8±0.3	5.5±0.4
17d18d	3605±376	7.0±0.6	2.8±0.3	5.5±0.5
17e18e	3502±340	6.8±0.6	2.9±0.3	5.3±0.5
23a24a	3292±317	6.3±0.6	2.6±0.3	5.8±0.6
23b24b	3345±346	6.4±0.7	2.5±0.3	6.0±0.6
23c24c	4925±369	9.5±0.9	1.5±0.1	9.9±0.9
23d24d	3723±311	7.2±0.7	2.0±0.2	7.5±0.8
23e24e	3633±318	7.0±0.7	2.0±0.2	7.5±0.7
23f24f	3826±319	7.4±0.7	1.8±0.1	8.2±0.8

The values are expressed as the mean ± SEM, n=3 independent experiments.
(A) = Bmax (fmol/mg protein) and Bmax shift obtained in [^{3}H]CCPA saturation binding experiments performed in the absence (K_D= 1.1 ± 0.1 nM; Bmax = 522±46 fmol/mg protein) or in the presence of 10 μM enhancers.
(B) = Ki values of CCPA in the presence of 10 μM tested compounds and CCPA shift = Ki(CCPA)/Ki(CCPA+10 μM enhancers) where the Ki of CCPA was 15.1± 1.6 nM.

FIGURE LEGENDS

Figure 1. Chemical structures of 2-amino-3-(hetero)aroyl-4,5-dialkythiophene derivatives **1-8** and *tert*-butyl 2-amino-3-(4-chlorobenzoyl)-4,5,7,8-tetrahydro-6*H*-thieno[2,3-*d*]azepine-6-carboxylate **9**.

Figure 2. Chemical structures of 2-amino-3-substituted-4-aryl-5-alkyl/aryl thiophene analogues **9-16**10-17.

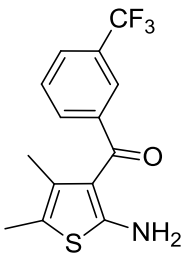
Figure 3. Chemical structures of 2-amino-3-(4-chlorobenzoyl)-4-[4-(aryl)piperazin-1-yl)methyl]thiophene derivatives **17a-17e**18a-e.

Figure 4. Chemical structures of 2-amino-3-(4-chlorobenzoyl)-4-substituted-5-aryl thiophene derivatives **18-22**19-23.

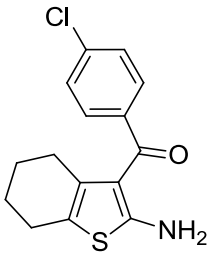
Figure 5. Chemical structures of 2-amino-3-(*p*-chlorobenzoyl)-4-neopentyl-5-aryl/arylethynylthiophene derivatives **23a-f**24a-f evaluated as allosteric modulators for A_1ARs.

Figure 6. A_1AR allosteric modulation and interaction with Gi proteins. Inhibitory effect of novel allosteric enhancers **23c-f**24c-f on cyclic AMP assay in hA_1CHO cells.

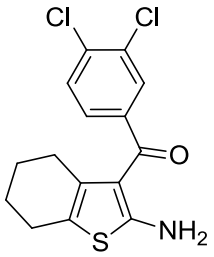
Figure 1.



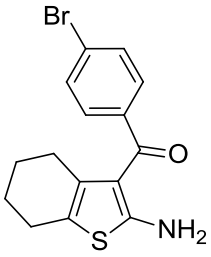
1 (PD 81,723)



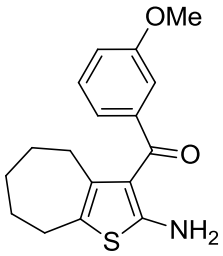
2 (T-62)



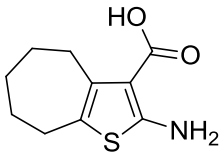
3 (LUF 5484)



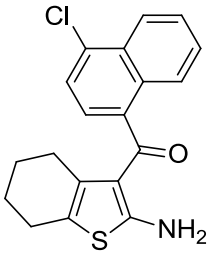
4



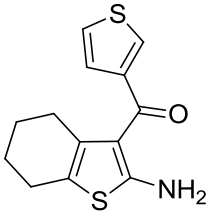
5



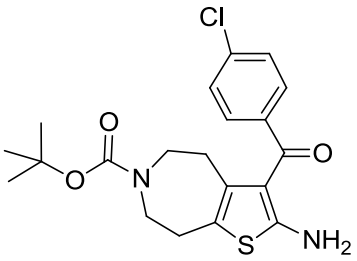
6



7



8



9 (VCP333)

Figure 2.

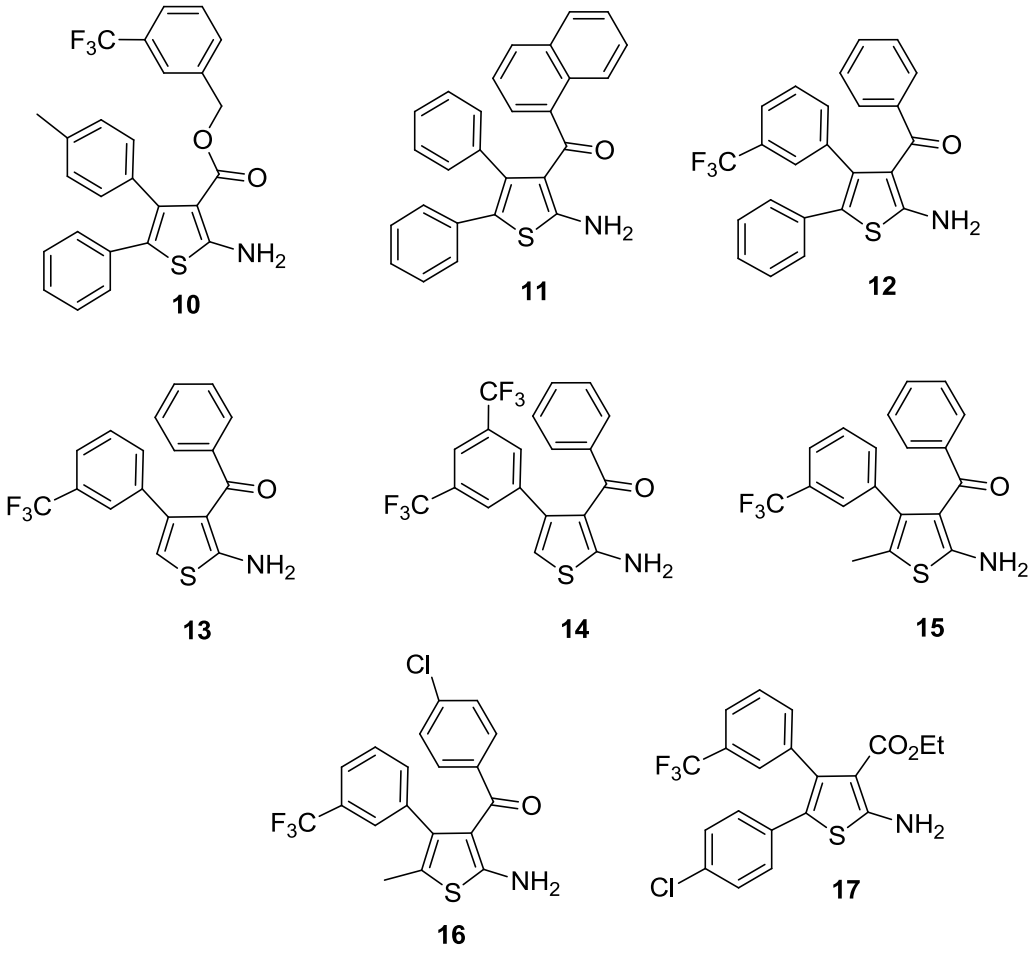
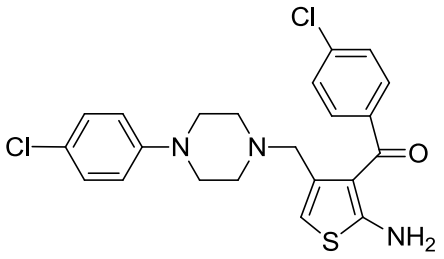
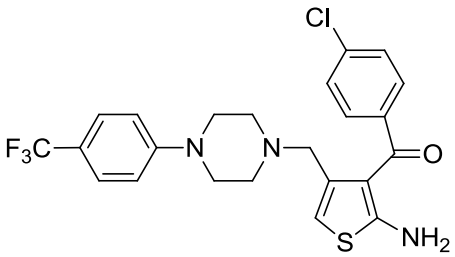

F3C
O
O
S
NH2
10
O
S
NH2
11
F3C
O
S
NH2
12
F3C
O
S
NH2
13
CF3
F3C
O
S
NH2
14
F3C
O
S
NH2
15
Cl
F3C
O
S
NH2
16
F3C
CO2Et
S
NH2
Cl
17

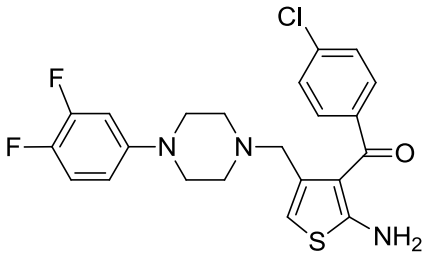
Figure 3.



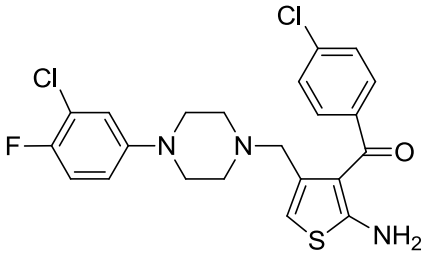
18a



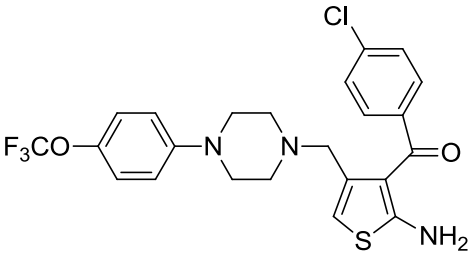
18b



18c

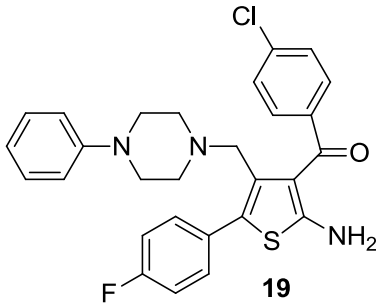


18d

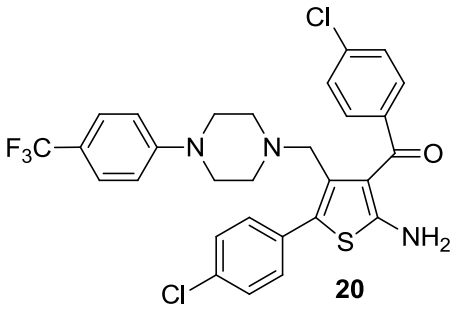


18e

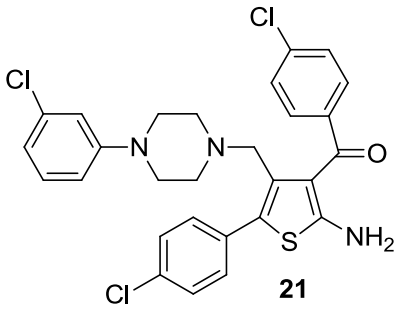
Figure 4.



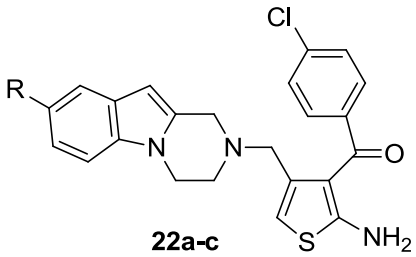
19



20

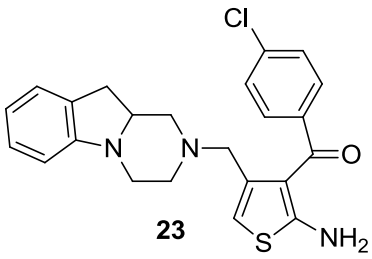


21



22a-c

22a; R=H
22b; R=F
22c; R=Cl



23

Figure 5.

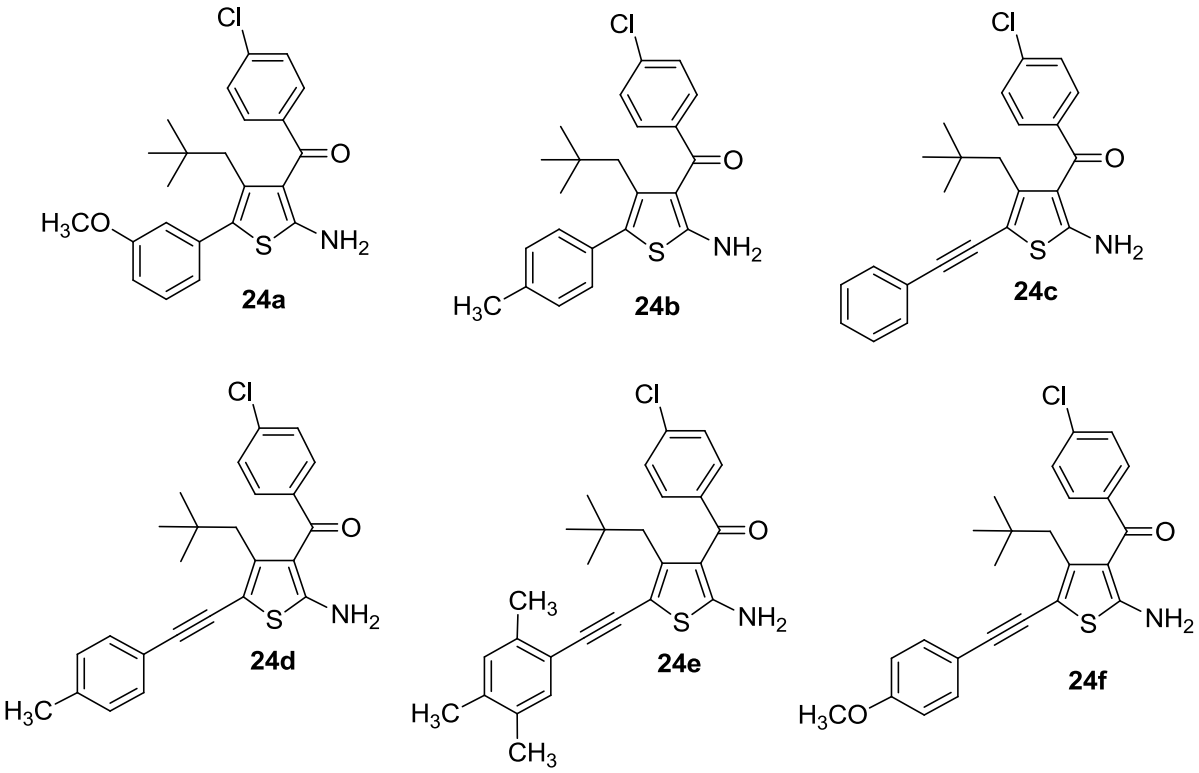
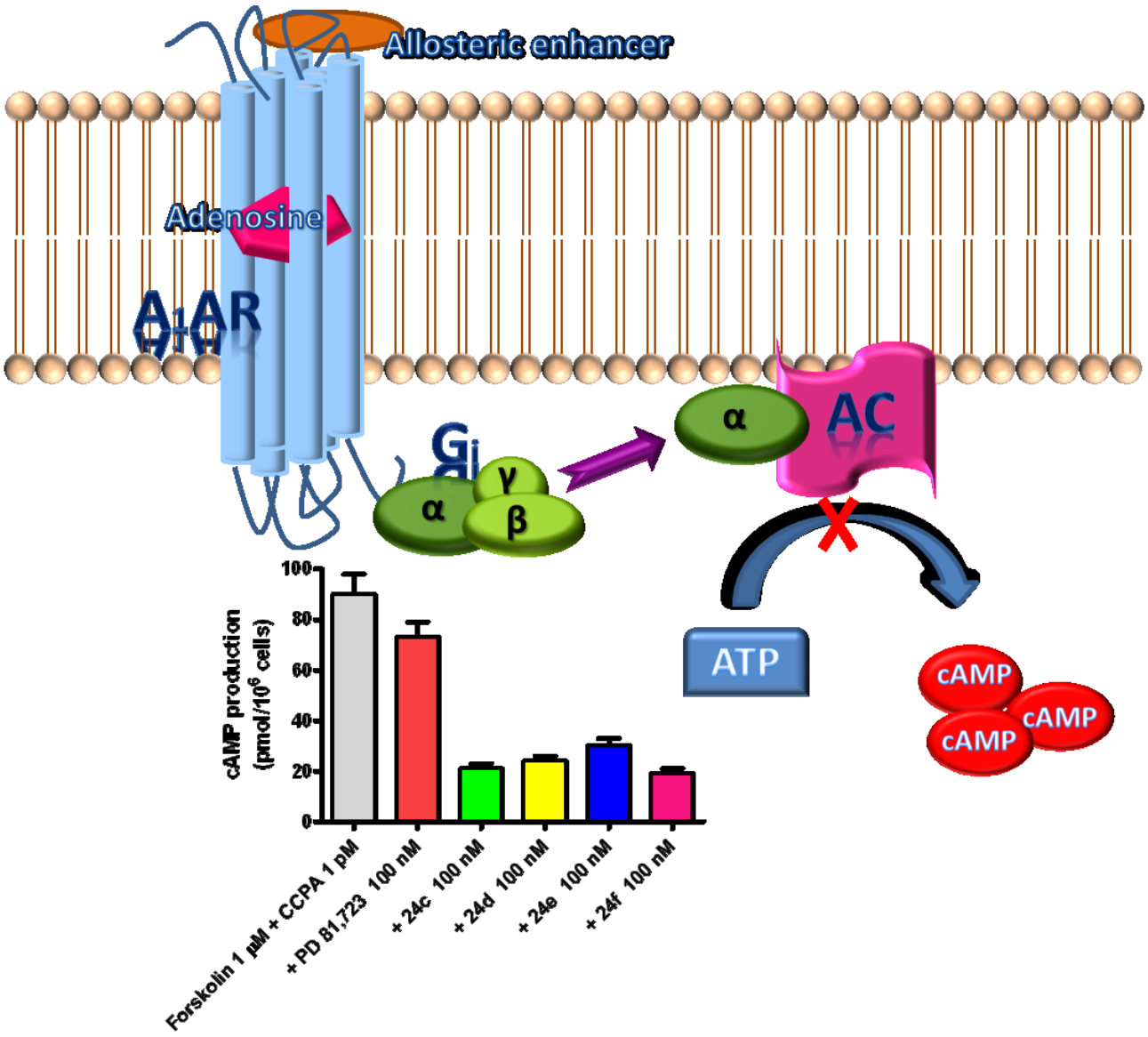

Cl
O
H3CO
S
NH2
24a
Cl
O
S
NH2
H3C
24b
Cl
O
S
NH2
24c
Cl
O
S
NH2
H3C
24d
Cl
O
CH3
S
NH2
H3C
CH3
24e
Cl
O
S
NH2
H3CO
24f

Figure 6


Allosteric enhancer
Adenosine
A1AR
Gi
α
γ
β
α
AC
ATP
cAMP
cAMP
cAMP
cAMP production (pmol/10^6 cells)
100
80
60
40
20
0
Forskolin 1 µM + CCPA 1 pM
+ PD 81,723 100 nM
+ 24c 100 nM
+ 24d 100 nM
+ 24e 100 nM
+ 24f 100 nM

BIBLIOGRAPHY

Papers of special note have been highlighted as: ■ of interest; ■■ of special interest.

[1] Burnstock G. Introductory overview of purinergic signalling. *Front. Biosci.* (Elite Ed) 3, 896-900 (2011).

[2] Fredholm BB, IJzerman AP, Jacobson KA, Linden J, Muller CE. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors-an update. *Pharmacol. Rev.* 63(1), 1-34 (2011).

[3] Fredholm BB. Adenosine receptors as drug targets. *Exp Cell Res* 316(8), 1284-1288 (2010).

[4] Lin H, Sassano MF, Roth BL, Shoichet BK. A pharmacological organization of G protein coupled receptors. *Nat. Methods* 10, 140-146 (2013).

[5] Schulte G, Fredholm BB. Signaling from adenosine receptors to mitogen activated protein kinases. *Cell Signal.* 15, 813-827 (2003).

[6] Chen JF, Eltzschig HK, Fredholm BB. Adenosine receptors as drug targets-what are the challenges? *Nat. Rev. Drug Discov.* 12(4), 265-286 (2013).

[7] Stone TW, Ceruti S, Abbracchio MP. Adenosine receptors and neurological disease: neuroprotection and neurodegeneration. *Handb. Exp. Pharmacol.* 193, 535-587 (2009).

[8] Burnstock G. Purinergic signalling: pathophysiology and therapeutic potential. *Keio J. Med.* 62(3), 63-73 (2013).

[9] Headrick JP, Ashton KJ, Rose'meyer RB, Peart JN. Cardiovascular adenosine receptors: expression, actions and interactions. *Pharmacol. Ther.* 140(1), 92-111 (2013).

[10] Dhalla AK, Shryock JC, Shreenivas R, Belardinelli L. Pharmacology and therapeutic application of A_1 adenosine receptor ligands. *Curr. Top. Med. Chem.* 3(4), 369-385 (2003).

[11] Gao Z-G, Jacobson KA. Emerging adenosine receptor agonists. *Expert. Opin. Emerg. Drugs* 12(3), 479-492 (2007).

[12] Lopes LV, Sebastião AM, Ribeiro JA. Adenosine and related drugs in brain diseases: present and future in clinical trials. *Curr. Top Med. Chem.* 11(8), 1087-1101 (2011).

[13] Schwartz TW, Holst B. Allosteric enhancers, allosteric agonists and ago-allosteric modulators: where do they bind and how do they act? *Trends Pharmacol. Sci.* 28(8), 366-373 (2007).

[14] Giorgi I, Nieri P. Adenosine A_1 modulators: a patent update (2008 to present). *Expert Opin. Ther. Pat.* 23(9), 1109-1121 (2013).

[14] Christopoulos A, Changeux JP, Catterall WA, *et al.* International union of basic and clinical pharmacology. XC. multisite pharmacology: recommendations for the nomenclature of receptor allosterism and allosteric ligands. *Pharmacol Rev.* 66 (4) 918-947 (2014).

[15] Milligan G, Smith NJ. Allosteric modulation of heterodimeric G protein-coupled receptors. *Trends Pharmacol. Sci.* 28, 615-620 (2007).

[16] Melancon BJ, Hopkins CR, Wood MR, Emmitte KA, Niswender CM, Christopoulos A, Conn PJ, Lindsley CW *et al.* Allosteric modulation of seven transmembrane spanning receptors: Theory, Practice, and Opportunities for Central Nervous System Drug Discovery. *J. Med. Chem.* 55(4), 1445-1464 (2012).

■ **A clear and detailed description of the concept of allosteric modulation.**

[17] Christopoulos A. Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nature Rev. Drug Discov.* 1(3), 198-210 (2002).

[18] Lane JR, Abdul-Ridha A, Canals M. Regulation of G protein-coupled receptors by allosteric ligands. *ACS Chem Neurosci.* 4, 527-534 (2013).

[19] Narlawar R, Lane JR, Doddareddy M, Lin J, Brussee J, IJzerman AP. Hybrid ortho/allosteric ligands for the adenosine A_1 receptor. *J. Med. Chem.* 53(8), 3028-3037 (2010).

[20] May LT, Leach K, Sexton PM, Christopoulos A. Allosteric modulation of G protein coupled receptors. *Annu. Rev. Pharmacol. Toxicol.* 47, 1-51 (2007).

[21] Valant C, Aurelio L, Urmaliya VB, White P, Scammells P J, Sexton PM, Christopoulos A *et al.* Delineating the mode of action of adenosine A_1 receptor allosteric modulators. *Mol. Pharmacol.* 78(3), 444-455 (2010).

[20] Kenakin TP. 7TM receptor allostery: putting numbers to shapeshifting proteins. *Trends in Pharmacol. Sci.* 30(9), 460-469 (2009).

[21] Conn PJ, Christopoulos A., Lindsey CW. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat. Rev. Drug Discov.* 8, 41-54 (2009).

[22] Kennedy DP, McRobb FM, Leonhardt SA, Purdy M, Figler H, Marshall MA, Chordia M, Figler R, Linden J, Abagyan R, Yeager M *et al.* The second extracellular loop of the adenosine A_1 receptor mediates activity of allosteric enhancers. *Mol. Pharmacol.* 85(2),301-309 (2014).

[23] Figler H, Olsson RA, Linden J. Allosteric enhancers of A_1 adenosine receptors increase receptor-G protein coupling and counteract guanine nucleotide effects on agonist binding. *Mol. Pharmacol.* 64(6), 1557-1564 (2003).

[23] Avlani VA, Gregory KJ, Morton CJ, Parker MW, Sexton PM, Christopoulos A. Critical role for the second extracellular loop in the binding of both orthosteric and allosteric G protein-coupled receptor ligands. *J. Biol. Chem.* 282, 25677-25686 (2007).

[24] Canals M, Lane JR, Wen A, Scammells PJ, Sexton PM, Christopoulos A. A Monod-Wyman-Changeux mechanism can explain G protein coupled receptor (GPCR) allosteric modulation. *J. Biol. Chem.* 287, 650-659 (2012).

[25] Pietra D, Borghini A, Breschi MC, Bianucci AM. Enhancer and competitive allosteric modulation model for G-protein coupled receptors. *J. Theor. Biol.* 267, 663-675 (2010).

[26] Christopoulos A, Kenakin T. G Protein-receptor coupling allosterism and complexing. *Pharmacol. Rev.* 54(2), 323-374 (2002).

[27] Peeters MC, Wisse LE, Dinaj A, Vroling B, Vriend G, Ijzerman AP. The role of the second and third extracellular loops of the adenosine A1 receptor in activation and allosteric modulation. *Biochem. Pharmacol.* 84, 76-87 (2012).

[28] Conn PJ, Christopoulosus A. Lindsey CW. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat. Rev. Drug Discov.* 8, 41-54 (2009).

[28] May LT, Leach K, Sexton PM, Christopoulos A. Allosteric modulation of G protein coupled receptors. *Annu. Rev. Pharmacol. Toxicol.* 47, 1-51 (2007).

[29] Bruns RF, Fergus JH. Allosteric enhancement of adenosine A_1 receptor binding and function by 2-amino-3-benzoylthiophenes. *Mol. Pharmacol.* 38(6), 939-949 (1990).

[30] Bruns RF, Fergus JH, Coughenour LL, Courtland GG, Pugsley TA, Dodd JH, Tinney FJ *et al.* Structure-activity relationships for enhancement of adenosine A_1 receptor binding by 2-amino-3-benzoylthiophenes. *Mol. Pharmacol.* 38(6), 950-958 (1990).

■■ **This article reports the discovery of the reference compound PD 81,723**

[31] Musser B, Mudumbi RV, Liu J, Olson RD, Vestal RE. Adenosine A_1 receptor-dependent and-independent effects of the allosteric enhancer PD 81,723. *J. Pharmacol. Exp. Ther.* 288(2), 446-454 (1999).

[32] Bhattacharya S, Linden J. Effects of long-term treatment with the allosteric enhancer, PD-81,723, on Chinese hamster ovary cells expressing recombinant human A_1 adenosine receptors. *Mol. Pharmacol.* 50(1), 104-111 (1996).

[33] Bhattacharaya S, Linden J. The allosteric enhancer, PD 81,723, stabilizes human A_1 adenosine receptor coupling to G proteins. *Biochim. Biophys. Acta* 1265(1), 15-21 (1995).

[34] Soudijin W, Wijngaarden I, Ijzerman AP. Allosteric modulation of G-protein-coupled receptors. *Expert Opin. Ther. Patents* 11(12), 1889-1904 (2001).

[35] IJzerman AP, Kouronakis A, van der Klein P. Allosteric modulators of G protein-coupled receptors. *Il Farmaco* 56(1-2), 67-71 (2001).

[36] Baraldi PG, Moorman AR, Aghazadeh Tabrizi M, Pavani MG. Romagnoli R. Allosteric modultors for the A_1-adenosine receptor. *Exp. Opin. Therap. Patents* 14(1), 71-79 (2004).

[37] May LT, Avlani VA, Sexton PM, Christopoulos A. Allosteric modulation of G protein-coupled receptors. *Curr. Pharm. Des.* 10(17), 2003-2013 (2004).

[38] Gao Z-G, Kim S-K, Ijzerman AP, Jacobson KA. Allosteric modulation of the adenosine family of receptors. *Mini Rev. Med. Chem.* 5(6), 545-553 (2005).

■■ **Exhaustive review concerning the structure-activity relationship of allosteric modulators at the A_1ARs.**

[39] Baraldi PG, Iaconinoto MA, Moorman AR, Carrion MD, Cara C-L, Preti D, López OC, Fruttarolo F, Aghazadeh Tabrizi M, Romagnoli R. *et al.* Allosteric enhancers for A_1 adenosine receptor. *Mini Rev. Med. Chem.* 7(6), 559-569 (2007).

[40] May LT, Leach K, Sexton PM, Christopoulos A. Allosteric modulation of G protein coupled receptors. *Annu. Rev. Pharmacol.Toxicol.* 47, 1-51 (2007).

[41] Göblyös A, IJzerman AP. Allosteric modulation of adenosine receptors. *Purinergic Signalling*. 5(1), 51-61 (2009).

■■ This article describes a wide panel of compounds available as allosteric modulators for A_1ARs.

[42] Romagnoli R, Baraldi PG, Aghazadeh Tabrizi M, Gessi S, Borea PA, Merighi S. Allosteric enhancers of A1 adenosine receptors: state of the art and new horizons for drug development. *Curr. Med. Chem.* 17(30), 3488-3502 (2010).

[43] Valant C, Aurelio L, Urmaliya VB, White P, Scammells PJ, Sexton PM, Christopoulos A. *et al.* Delineating the mode of action of adenosine A_1 receptor allosteric modulators. *Mol Pharmacol.* 78(3), 444-455 (2010).

[44] Göblyös A, IJzerman AP. Allosteric modulation of adenosine receptors. *Biochim. Biophys. Acta* 1808(5), 1309-1318 (2011).

[45] Jacobson KA, Gao Z-G, Göblyös A, IJzerman AP. Allosteric modulation of purine and pyrimidine receptors. *Adv Pharmacol.* 61, 187-220 (2011).

[46] van der Klein PAM, Kourounakis AP, IJzerman AP. Allosteric modulation of the adenosine A_1 receptor. Synthesis and biological evaluation of novel 2-amino-3-benzoylthiophenes as allosteric enhancers of agonist binding, *J. Med. Chem.* 42(18), 3629-3635 (1999).

[47] Childers SR, Li X, Xiao R, Eisanach JC. Allosteric modulation of adenosine A_1 receptor coupling to G-proteins in brain. *J. Neurochem.* 93(3), 715-723 (2005)

[48] Li X, Conklin D, Ma W, Zhu X, Eisenach JC. Spinal noradrenergic activation mediates allodynia reduction from an allosteric adenosine modulator in a rat model of neuropathic pain. *Pain* 97(1-2), 117-125 (2002).

[49] Li X, Conklin D, Pan H-L, Eisenach JC. Allosteric adenosine receptor modulation reduces hypersensitivity following peripheral inflammation by a central mechanism. *J. Pharmacol. Exp. Ther.* 305(3), 950-955 (2003).

[50] ClinicalTrials.gov Identifier: NCT00809679.

[51] Baraldi PG, Zaid AN, Lampronti I, Fruttarolo F, Pavani MG, Tabrizi MA, Shryock JC, Leung E, Romagnoli R. *et al.* Synthesis and biological effects of a new series of 2-amino-3-benzoylthiophenes as allosteric enhancers of A_1 adenosine receptor. *Bioorg. Med. Chem. Lett.* 10(17), 1953-1957 (2000).

[52] Tranberg CE, Zickgraf A, Giunta BN, Luetjens H, Figler H, Murphree LJ, Falke R, Fleischer H, Linden J, Scammells PJ, Olsson RA. *et al.* 2-Amino-3-aroyl-4,5-alkylthiophenes: agonist allosteric enhancers at human A_1 adenosine receptors. *J. Med. Chem.* 45(2), 382-389 (2002).

[53] Nikolakopoulos G, Figler H, Linden J, Scammells PJ. Aminothiophene-3-carboxylates and carboxamides as adenosine A_1 receptor allosteric enhancers. *Bioorg. Med. Chem.* 14(7), 2358-2365 (2006).

[54] Baraldi PG, Romagnoli R, Pavani MG, Nuñez MC, Aghazadeh Tabrizi M, Shryock JC, Leung E, Moorman AR, Uluoglu C, Iannotta V, Merighi S, Borea PA. *et al.* Synthesis and

biological effects of novel 2-amino-3-naphthoylthiophenes as allosteric enhancers of the A_1 adenosine receptor. *J. Med. Chem.* 46(5), 794-809 (2003).

[55] Baraldi PG, Pavani MG, Shryock JC, Moorman AR, Iannotta V, Borea PA, Romagnoli R. *et al.* Synthesis of 2-amino-3-heteroaroylthiophenes and evaluation of their activity as potential allosteric enhancers at the human A_1 receptor. *Eur. J. Med. Chem.* 39(10), 855-865 (2004).

[56] Butcher A, Scammels PJ, White PJ, Devine SM, Meyer RBB. An allosteric modulator of the adenosine A_1 receptor improves cardiac function following ischaemia in murine isolated hearts. *Pharmaceuticals* 6, 546-556 (2013).

[5657] Luetjens H, Zickgraf A, Figler H, Linden J, Olsson RA, Scammells PJ. 2-Amino-3-benzoylthiophene allosteric enhancers of A_1 adenosine agonist binding: new 3,4-, and 5-modifications. *J. Med. Chem.* 46(10), 1870-1877 (2003).

[5758] Aurelio L, Figler H, Flynn BL, Linden J, Scammells PJ. 5-Substituted 2-aminothiophenes as A_1 adenosine receptor allosteric enhancers. *Bioorg. Med. Chem.* 16(3), 1319-1327 (2008).

[5859] Aurelio L, Valant C, Sexton PM, Christopoulos A, Scammells PJ. Allosteric modulators of the adenosine A_1 receptor: synthesis and pharmacological evaluation of 4-substituted 2-amino-3-benzoylthiophenes. *J. Med. Chem.* 52(14), 4543-4547 (2009).

[5960] Valant C, Aurelio L, Devine SM, Ashton TD, White JM, Sexton PM, Christopoulos A, Scammells PJ. *et al.* Synthesis and characterization of novel 2-amino-3-benzoylthiophene derivatives as biased allosteric agonists and modulators of the adenosine A_1 receptor. *J. Med. Chem.* 55(5), 2367-2375 (2012).

[6061] Aurelio R, Christopoulos A, Flynn BL, Scammells PJ, Sexton PM, Valant C. The synthesis and biological evaluation f 2-amino-4,5,6,7,8,9-hexaydrocycloocta[b]thiophenes as allosteric modulators of the A_1 adenosine receptor. *Bioorg. Med. Chem. Lett.* 21(12), 3704-3707 (2011).

[6162]Romagnoli R, Baraldi PG, Carrion MD, Cara CL, Cruz-Lopez O, Iaconinoto MA. Preti D, Shryock JC, Moorman AR, Vincenzi F, Varani K, Borea PA. *et al.* Synthesis and biological evaluation of 2-amino-3-(4-chlorobenzoyl)-4-[*N*-(substituted) piperazin-1-yl]thiophenes as potent allosteric enhancers of the A_1 adenosine receptor. *J. Med. Chem.* 51(18), 5875-5879 (2008).

[6263]Romagnoli R, Baraldi PG, Carrion MD, Cara CL, Cruz-Lopez O, Salvador MK, Preti D, Tabrizi MA, Shryock JC, Moorman AR, Vincenzi F, Varani K, Borea P. A. *et al.* Structure-activity relationships of 2-amino-3-aroyl-4-[(4-arylpiperazin-1-yl)methyl]thiophenes. Part 2. Probing the influence of diverse substituents at the phenyl of the arylpiperazine moiety on allosteric enhancer activity at the A_1 adenosine receptor. *Bioorg. Med. Chem.* 20(2), 996-1007 (2012).

[6364]Romagnoli R, Baraldi PG, Carrion MD, Cara CL, Cruz-Lopez O, Salvador MK, Preti D, Tabrizi MA, Moorman AR, Vincenzi F, Borea PA, Varani K.. *et al.* Synthesis and biological evaluation of 2-amino-3-(4-chlorobenzoyl)-4-[(4-arylpiperazin-1-yl)methyl]-5-substituted thiophenes. Effect of the 5-modification on allosteric enhancer activity at the A_1 adenosine receptor. *J. Med. Chem.* 55(17), 7719-7735 (2012).

[6465]Vincenzi F, Targa M, Romagnoli R, Merighi S, Gessi S, Baraldi PG, Borea PA, Varani K. *et al.* TRR 469, a potent A_1 adenosine receptor allosteric modulator, exhibits anti-nociceptive properties in acute and neurophatic pain models in mice. *Neuropharmacology* 81, 6-14 (2014).

[6566]Romagnoli R, Baraldi PG, Carrion MD, Lopez Cara C, Kimatrai Salvador M, Preti D, Aghazadeh Tabrizi M, Moorman AR, Vincenzi F, Borea PA, Varani K. *et al.* Synthesis and biological effects of novel 2-amino-3-(4-chlorobenzoyl)-4-substituted thiophenes as allosteric enhancers of the A_1 adenosine receptor. *Eur. J. Med. Chem.* 67, 409-427 (2013).

[6667]Romagnoli R, Baraldi PG, Carrion MD, Cruz-Lopez O, Cara CL, Saponaro G, Preti D, Tabrizi MA, Baraldi S, Moorman AR, Vincenzi F, Borea PA, Varani K. *et al.* Synthesis and

Biological Evaluation of Novel 2-Amino-3-Aroyl-4-Neopentyl-5-Substituted Thiophene Derivatives as Allosteric Enhancers of the A_1 Adenosine Receptor. *Bioorg. Med. Chem.* 22(1), 148-166 (2014).

[6768]Romagnoli R, Baraldi PG, IJzerman AP, Massink A, Cruz-Lopez O, Lopez-Cara LC, Saponaro G, Preti D, Aghazadeh Tabrizi M, Baraldi S, Moorman AR, Vincenzi F, Borea PA, Varani K. *et al.* Synthesis and Biological Evaluation of Novel Allosteric Enhancers of the A_1 Adenosine Receptor Based on 2-Amino-3-(4'-Chlorobenzoyl)-4-Substituted-5-Arylethynyl Thiophene. *J. Med. Chem.* 57(18), 7673-7686 (2014).

[6869]Chordia MD, Murphree LJ, Macdonald TL, Linden J, Olsson RA. 2-Aminothiazoles: A new class of agonist allosteric enhancers of A_1 adenosine receptors. *Bioorg. Med. Chem. Lett.* 12(12), 1563-1566 (2002).

[6970]Ferguson GN, Valant C, Horne J, Figler H, Flynn BL, Linden J, Chalmers KL, Sexton PM, Christopoulos A, Scammells PJ. *et al.* 2-Aminothienopyridazines as novel adenosine A_1 receptor allosteric modulators and antagonists. *J. Med. Chem.* 51(19), 6165-6172 (2008).

[7071]van den Nieuwendijk AMCH, Pietra D, Heitman L, Göblyös A, IJzerman AP. Synthesis and biological evaluation of 2,3,5-substituted [1,2,4]thiadiazoles as allosteric modulators of adenosine receptors. *J. Med. Chem.* 47(3), 663-672 (2004).